METHODS OF TREATING INFLAMMATORY SKIN DISEASES

Statement of Related Applications

[0001] The application is a divisional of Application No. 09/773,877, filed 31 Jan 2001, now allowed, which is the National Stage of International Application No. PCT/US00/14142, filed 23 May2000, which claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/138,133, filed 8 June 1999.

BACKGROUND

Field of the Invention

[0002] This invention relates to methods of treating inflammatory skin diseases with an antagonist of vascular endothelial cell growth factor (VEGF). More specifically the invention relates to methods of using a VEGF antagonist in the treatment of psoriasis.

Statement of Related Art

[0003] Persistent angiogenesis may cause or exacerbate certain diseases such as psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas, diabetic retinopathy and neovascular glaucoma. An inhibitor of VEGF activity would be useful as a treatment for such diseases and other VEGF-induced pathological angiogenesis and vascular permeability conditions, such as tumor vascularization. The present invention relates to a VEGF inhibitor that is based on the VEGF receptor Flt1.

[0004] Plasma leakage, a key component of inflammation, occurs in a distinct subset of microvessels. In particular, in most organs plasma leakage occurs specifically in the venules. Unlike arterioles and capillaries, venules become leaky in response to numerous inflammatory mediators including histamine, bradykinin, and serotonin. One characteristic of inflammation is the plasma leakage that results from intercellular gaps that form in the endothelium of venules. Most experimental models of inflammation indicate that these intercellular gaps occur between the endothelial cells of postcapillary and collecting venules (Baluk et al. (1998) Am. J. Pathol. 152:1463-76). It has been shown that certain lectins may be used to reveal features of focal sites of plasma

leakage, endothelial gaps, and finger-like processes at endothelial cell borders in inflamed venules (Thurston et al. (1996) Am. J. Physiol, 271:H2547-62). In particular, plant lectins have been used to visualize morphological changes at endothelial cell borders in inflamed venules of, for example, the rat trachea. Lectins, such as conconavalin A and ricin, that bind focally to inflamed venules reveal regions of the subendothelial vessel wall exposed by gaps that correspond to sites of plasma leakage (Thurston et al. (1996) *supra*).

[0005] US Patent 6,011,003 (Metris Therapeutics Limited) discloses an altered, soluble form of Flt polypeptide being capable of binding to VEGF and thereby exerting an inhibitory effect thereon, the polypeptide comprising five or fewer complete immunoglobulin domains. US Patent 5,712,380 (Merck & Co.) discloses vascular endothelial cell growth factor (VEGF) inhibitors that are naturally occurring or recombinantly engineered soluble forms with or without a C-terminal transmembrane region of the receptor for VEGF. WO 97/44453 (Genentech, Inc.) discloses novel chimeric VEGF receptor proteins comprising amino acid sequences derived from the vascular endothelial growth factor (VEGF) receptors Flt1 and KDR, including the murine homologue to the human KDR receptor FLK1, wherein said chimeric VEGF receptor proteins bind to VEGF and antagonize the endothelial cell proliferative and angiogenic activity thereof. WO 97/13787 (Toa Gosei Co., LTD.) discloses a low molecular weight VEGF inhibitor usable in the treatment of diseases accompanied by neovascularization such as solid tumors.

BRIEF SUMMARY OF THE INVENTION

[0005] In a first aspect, the invention features a method of treating inflammatory skin disease in a mammal , comprising administering a VEGF antagonist to the mammal. In a preferred embodiment, the VEGF antagonist is a fusion polypeptide capable of binding a VEGF polypeptide comprising (a) a VEGF receptor component operatively linked to (b) a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion polypeptide and consists essentially of a the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor. In a specific embodiment, the first VEGF receptor is Flt1, the second VEGF receptor is Flk1 or Flt4. In specific embodiments, the VEGF antagonist is a fusion polypeptide

selected from the group consisting of Flt-1(1-3)-Fc, Flt-1(1-$3_{R->N}$)-Fc (described in Fig. 16A-C of parent application USSN 09/773,877), Flt-1(1-$3_{\Delta B}$)-Fc (described in Fig. 13A-D of parent application USSN 09/773,877, herein specifically incorporated by reference in its entirety), Flt-1(2-$3_{\Delta B}$)-Fc (described in Fig. 14A-C of parent application USSN 09/773,877), Flt-1(2-3)-Fc (described in Fig. 15A-C of parent application USSN 09/773,877), Flt-1D2-VEGFR3D3-FcΔC1(a), Flt-1D2-Flk-1D3-FcΔC1(a), and VEGFR1R2-FcΔC1(a). In further preferred embodiments, Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of Ig domain 3 of the extracellular domain of the second VEGF receptor. In still another preferred embodiment, the Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of Ig domain 3 of the extracellular domain of the second VEGF receptor. In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain. In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In a most specific embodiment, the VEGF antagonist is a VEGF trap comprised of VEGFR1R2-FcDC1(a). In a preferred embodiment, the mammal is a human.

[0006] In a second aspect, the invention features a method of reducing the severity of a psoriatic lesion in a mammal comprising administering a VEGF antagonist to the mammal.

[0007] In a third aspect, the invention features a method of minimizing the extent of hyperproliferation of keratinocytes associated with psoriasis in a mammal comprising administering a VEGF antagonist to the mammal.

[0008] In a fourth aspect, the invention features a method of minimizing the extent of epidermal hyperplasia associated with psoriasis in a mammal comprising administering a VEGF antagonist to the mammal.

[0009] In a fifth aspect, the invention features a method of reversing epidermal hyperplasia associated with psoriasis in a mammal, comprising administering a VEGF antagonist to the mammal.

[0010] In a sixth aspect, the invention features methods of treating parakeratosis and treating microabcess associated with psoriasis in a human, comprising administering a VEGF antagonist to the human.

[0011] In a seventh aspect, the invention features a method of decreasing reteridges associated with psoriasis in a human comprising administering a VEGF antagonist to the human.

[0012] In an eighth aspect, the invention features a method of preventing the infiltration of lymphocytes from the dermis into the epidermis of a human comprising administering VEGFR1R2-FcDC1(a) to the human.

[0013] In preferred embodiments of the invention the administration is topical administration, subcutaneous administration, or perhaps intramuscular, intranasal, intrathecal, intraarterial, intravenous, transvaginal, transdermal, or transanal administration.

[0014] In a ninth aspect, the invention features a method of enhancing wound healing in a human comprising administering a VEGF antagonist to the human. Another preferred embodiment is a method of enhancing wound healing in a human comprising administering VEGFR1R2-FcDC1(a) to the human. In preferred embodiments of the invention the administration is topical administration, subcutaneous administration, or perhaps intramuscular, intranasal, intrathecal, intraarterial, intravenous, transvaginal, transdermal, or transanal administration.

BRIEF DESCRIPTION OF THE FIGURES

[0015] Fig. 1. The ability of Flt1D2.Flk1D3.FcΔC1(a) to inhibit HT-1080 fibrosarcoma tumor growth *in vivo*. Every other day or 2 times per week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) at 25mg/Kg significantly decreases the growth of subcutaneous HT-1080 fibrosarcoma tumors.

[0016] Fig. 2. The ability of Flt1D2.Flk1D3.FcΔC1(a) to inhibit C6 glioma tumor growth *in vivo*. Every other day or 2 times a week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) significantly decreases the growth of subcutaneous C6 glioma tumors at doses as low as 2.5mg/Kg.

[0017] Fig. 3 VEGF-Induced Uterine Hyperpermeability. Subcutaneous injection of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at 1hr after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight.

[0018] Fig. 4A-B. Assessment of Corpus Luteum Angiogenesis Using Progesterone as a Readout. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at 1hr. after PMSG injection resulted in a complete inhibition of the progesterone induction on day 4.

[0019] Fig. 5. Gross phenotype of K14VEGF transgenic mice. Six months after birth, the mice develop significant skin lesions on the ears and scalp. The skin becomes red, edematous, and

profoundly scaling (parakeratosis and hyperkeratosis) to the point of generalized desquamation.

[0020] Fig. 6A-C. Histology of ear skin from K14VEGF transgenic mice (hematoxylin and eosin stained sections). Fig. 6A: Control, nontransgenic wildtype mouse. Fig. 6B: K14VEGF transgenic mouse at three months of age. Fig. 6C: K14VEGF transgenic mouse at six months of age. Note thickening of epidermis with increased hyperkeratosis and parakeratosis (40X).

[0021] Fig. 7. Rete ridge formation in relatively mature psoriatic-like lesions in K14VEGF transgenic mice (Masson's Trichrome staining). K14VEGF transgenic mouse at 6 months of age has developed dramatic rete ridge structures, some of which are fused at the base (4x).

[0022] Fig. 8A-B. Formation of microabscesses in relatively mature psoriatic-like lesions in K14VEGF transgenic mice. (Hematoxylin and eosin staining of skin sections from K14VEGF transgenic mice at 6 months of age.) Fig. 8A: Monro microabscess. Fig. 8B: Kogoj microabscess.

[0023] Fig. 9A-P: Effect of VEGFR1R2-FcDC1(a) in an animal model of psoriasis. A K14VEGF transgenic mouse with severe skin lesions was injected with VEGFR1R2-FcDC1(a) (25 mg/kg) on day 0 (Fig. 9A-D), day 3 (Fig. 9E-H), day 7 (Fig. 9I-L), and day10 (Fig. 9M-P), and photographed after each injection.

DETAILED DESCRIPTION OF THE INVENTION

[0024] Before the present methods are described, it is to be understood that this invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only the appended claims.

[0025] Psoriasis is a chronic skin disease characterized by red patches that are covered with white scales and is often accompanied by varying degrees of discomfort. The disease is not contagious; however, its cause and mechanism have not yet been determined. Because of the formation of unsightly skin lesions and eruptions, psoriasis often has a negative psychological impact on its sufferers. Among people in Western countries, approximately 2-3% of the total population suffers from the disease. Various classifications have been proposed for psoriasis, but it is generally classified into psoriasis vulgaris, pustular psoriasis, psoriatic arthritis, guttate psoriasis, and the like.

Of these, psoriasis vulgaris is the major type and accounts for 80 to 90% of all instances of the disease.

[0026] Psoriasis is considered to be a multicausal hereditary disease which is often triggered by the action of various non-genetic factors such as injury, infection, drugs, food, climate, and stress. Furthermore, psoriasis is known to be associated with certain histocompatibility antigens (HLA). In fact, studies have linked certain types of psoriasis with specific HLAs. For example, Tiilikainen et al. (1980) Br. J. Dermatol. 102:179-84, have reported that the prevalence of histocompatibility antigen HLA-Cw6 is 72.7% in twenty-two patients with the guttate form of psoriasis and 45.9% in thirty-seven patients with the vulgaris form of psoriasis. Thus, psoriasis clearly is a disease with a genetic basis in its cause.

[0027] There are two characteristic symptoms of psoriasis including 1) an inflammatory response common to that caused by other superficial skin diseases and 2) a tendency toward abnormal growth of the cuticle of the skin. The inflammatory response is characterized by vascular permeability, T-lymphocyte hypermigration, and release of the T-helper type I (THI) cytokine into the epidermis (Nickoloff et al. (1999) Arch Dermatol Sep;135(9):1104-10). The abnormal cuticle growth is characterized by epidermal acanthosis and rete ridge formation in more mature psoriasis. In more advanced psoriatic lesions, confluent parakeratosis with aberrantly differentiated keratinocytes containing nuclei in stratum corneum and microabcesses with neutrophils arranged in tiers within the confluent parakeratotic cornified layer (Altman et al. (1999) Seminars Cutaneous Med. Surg. 18:25-35) often develop. These are the key features for the clinical diagnosis of psoriasis.

[0028] The therapeutic methods currently available to treat psoriasis include the control of the hyperproliferation of epidermal cells; control of the inflammatory response; promotion of immunomodulation; and avoidance of infection by bacteria and fungi. The following is a summary of the therapeutic methods that are generally utilized: (1) External and internal use of adrenocortical hormone - The external or topical use of a steroid has the immediate effect of reducing the symptoms of psoriasis, particularly the reduction of eruptions. However, administration of adrenocortical hormone over long periods of time increases resistance and tolerance buildup, so that the dose must be increased, or stronger drugs must be used, in order to obtain an acceptable therapeutic effect. In addition, when the psoriatic lesion occurs over a relatively large area, it cannot be completely cured

by this method alone and, therefore, must be combined with other therapies; (2) Photochemotherapy - This method consists of administering psoralen in the form of an external or internal preparation and applying longwave ultraviolet rays to the affected region. Unfortunately, not all types of psoriasis can be treated by this method; (3) Phototherapy (UV Irradiation) - While this mode of treatment is often effective, over time it has the undesirable side effect of causing accelerated aging of the skin. In addition, there is the risk of inducing carcinogenesis; (4) External use of coal tar - Coal tar suppresses the growth of cells so that the psoriatic lesion diminishes over a short period of time and a relatively long remission period may be achieved. However, occasionally, other skin disorders can result such as stimulant dermatitis and folliculitis (tar acne); (5) Administration of methotrexate - Methotrexate is an antagonist against folic acid, which is active in inhibiting the growth of cells. The use of methotrexate is effective for treating pustular psoriasis. Unfortunately, the use of methotrexate for a long period of time causes adverse effects such as disturbances in liver function, suppression of myeloproliferation, and loss of reproductive function; (6) Administration of retinoid - Retinoid is considered to have an immunomodulation effect in that it may control the abnormal cornification of epidermal cells and increased leukocyte migration. The internal administration of retinoid-based therapeutics is particularly effective for treating pustular psoriasis and psoriatic erythroderma. However, retinoid can exhibit adverse effects such as a decrease in the thickness of the skin and the visible mucous membranes. Furthermore, abnormal levels of serum lipoprotein are occasionally observed. Importantly, because retinoid is teratogenic and likely to accumulate and remain inside the body for a long period of time, the administration of retinoid to people of childbearing age is avoided, thus limiting the patient population to those who are beyond childbearing age or who are suffering from intractable psoriasis; and (7) Cyclosporin A - an immunosuppressant that is often used by physicians for treating psoriasis. The major disadvantage of cyclosporin A as a treatment for psoriasis is that it is a general immunosuppressant, thus making patients more vulnerable to infection or other bacterial or viral diseases.

[0029] Recent studies have shown that the growth factor VEGF is upregulated in psoriatic lesions (Detmar et al. (1994) J Exp Med 1;180(3):1141-6). However, to date there are no data that describe what role the overexpression of VEGF may have in either the development of or the progression of psoriasis. It is known that VEGF causes vascular permeability, increased microvascular density and

enhanced leukocyte rolling and adhesion (Detmar et al. (1998) J. Invest. Dermatol. 111(1):1-6). Increased expression of VEGF has also been identified in chronic inflammatory dermatoses, including bullous pemphigoid, dermatitis herpetiformis, and erythema multiforme, all of which are characterized by hyperpermeable dermal microvessels and pronounced papillary dermal edema (Brown et al. (1995) J. Invest. Dermatol. 104(5):744-9).

[0030] While there are therapies available to treat psoriasis, most of these available therapies are less than ideal due to the severity of their side effects, the eventual development of resistance, and/or limitations on suitable patient populations, thus rendering clear the need for new safe and effective treatments for psoriasis. To satisfy this need, Applicants have discovered a new and novel method of treating psoriasis, such method utilizing a novel protein molecule that is able to reverse psoriatic-like lesions in a relatively short period of time with no apparent side effects during the course of treatment.

[0031] Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

[0032] The following example is put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: Modified Flt1 receptor vector construction

[0033] The rationale for constructing modified versions of the Flt1 receptor (also known as VEGFR1) was based on the observation that the protein sequence of Flt1 was highly basic, and was therefore likely to stick to extracellular matrix (ECM). The highly basic nature of Flt1 probably

explains why unmodified Flt1(1-3)-Fc (described in USSN 09/773,877, the specification of which is herein specifically incorporated by reference) has poor pharmacokinetics that make it difficult to use as a therapeutic agent. The chemically modified form of 40 fold molar excess acetylated Flt1(1-3)-Fc, hereinafter termed A40, exhibited a greatly improved pharmacokinetic (PK) profile over the non-acetylated Flt1(1-3)-Fc. Therefore, attempts were made to engineer DNA molecules that could be used to recombinantly express modified forms of a Flt1 receptor molecule that would possess the improved PK profile exhibited by A40 and still maintain the ability to bind tightly to VEGF.

[0034] It is known in the literature that the first Ig domain of Flt1 (which has a net charge of +5 at neutral pH) is not essential for tight binding to VEGF, so this domain was deleted. The third Ig domain (having a net charge of +11) is not essential for binding, but confers higher affinity for VEGF than the second Ig domain, so instead of deleting it entirely, it was replaced with the equivalent domains of the Flt1 receptor relatives Flk1 (also known as VEGFR2) and Flt4 (also known as VEGFR3). These chimeric molecules (denoted R1R2 (Flt1.D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) and R1R3 (Flt1D2.VEGFR3D3-FcΔC1(a) and VEGFR1R3-FcΔC1(a) respectively, wherein R1 and Flt1D2 = Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3 = Ig domain 3 of Flk1 (VEGFR2); and R3 and VEGFR3D3 = Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to ECM, as judged by an *in vitro* ECM binding assay as described *infra* , had greatly improved PK as described *infra*. In addition, these molecules were able to bind VEGF tightly and block phosphorylation of the native Flk1 receptor expressed in endothelial cells (described in USSN 09/773,877, the specification of which is herein specifically incorporated by reference).

[0035] (a) Construction of the expression plasmid pFlt1D2.Flk1D3.FcΔC1(a). Expression plasmids pMT21.Flt1(1-3).Fc (6519bp) and pMT21.Flk-1(1-3).Fc (5230bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAG TCCGGAGGTAGACCTTTCGTAGAGATG-3') (SEQ ID NO:1); 3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3') (SEQ ID NO:2). The 5'

amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:3) (corresponding to amino acids 27-33 of Fig. 21A-21C of USSN 09/773,877). The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of Fig. 21A-21C of USSN 09/773,877) and continuing into VVLS (SEQ ID NO:4)(corresponding to amino acids 127-130 of Fig. 21A-21C of USSN 09/773,877) of Flk1.

[0036] For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows: 5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATGG-3') (SEQ ID NO:5) 3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGACAAATG-3') (SEQ ID NO:6). The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO:7)(corresponding to amino acids 223-228 of Fig. 21A-21C of USSN 09/773,877), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Fig. 21A-21C of USSN 09/773,877. After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described in USSN 09/773,877) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in Fig. 21A-21C of USSN 09/773,877.

[0037] (b) Construction of the expression plasmid pFlt1D2VEGFR3D3FcΔC1(a). The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin resistance and an Fc-tagged

version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGA CCTTT CGTAGAGATG-3')(SEQ ID NO:8); 3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAG CTGGATATCTATGATTGTATTGGT) (SEQ ID NO:9). The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:10) (corresponding to amino acids 27-33 of Fig. 22A-22C of USSN 09/773,877). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO:11) of Flt1 (corresponding to amino acids 123-126 of Fig. 22A-22C of USSN 09/773,877) and continuing into IQLL (SEQ ID NO:12) of VEGFR3 (corresponding to amino acids 127-130 of Fig. 22A-22C of USSN 09/773,877). For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows: 5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA) 3' (SEQ ID NO:13): R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG) (SEQ ID NO:14). Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows: 5':Flt1D2.VEGFR3D3.s (TCATAGATATCCAGCTGTTG CCCAGGAAGTCGCTGGAG) (SEQ ID NO:15); 3': VEGFR3D3/srf.as (GATAATGCCC GGGCCATTTTCATGCACAATGACCTCGGT) (SEQ ID NO:16). The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (SEQ ID NO:17) (corresponding to amino acids 221-226 of Fig. 22A-22C of USSN 09/773,877), followed by a bridging sequence that includes a recognition sequence

for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Fig. 22A-22C of USSN 09/773,877.

[0038] After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1ΔB2.Fc (described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 693bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)ΔB2-FcΔC1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.FcΔC1(a) chimeric molecule is set forth in Fig. 22A-22C of USSN 09/773,877.

Example 2: Extracellular Matrix Binding (ECM) Binding Assay

[0039] ECM-coated plates (Becton Dickinson catalog # 35-4607) were rehydrated with warm DME supplemented with glutamine (2mM), 100U penicillin, 100U streptomycin, and 10% BCS for at least 1 hr. before adding samples. The plates were then incubated for 1 hr. at room temperature with varying concentrations of Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) starting at 10nM with subsequent 2-fold dilutions in PBS plus 10% BCS. The plates were then washed 3 times with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10% BCS) for 1 hr. at room temperature. The plates were then washed 4 times with PBS 0.1% Triton-X and alkaline phosphatase buffer/pNPP solution (Sigma) was added for color development. Plates were read at l = 405-570nm. The results of this experiment demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

Example 3: Transient expression of pFlt1D2.Flk1D3.FcΔC1(a) in CHO-K1 (E1A) cells

[0040] A large scale (2L) culture of E. coli DH10B cells carrying the pFlt1D2.Flk1D3.FcΔC1(a) plasmid described *supra* in Example 1(a) was grown overnight in Terrific Broth (TB) plus 100μg/ml ampicillin. The next day, the plasmid DNA was extracted using a QIAgen Endofree Megaprep kit following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined by standard techniques using a UV spectrophotometer and fluorometer. The plasmid DNA was verified by standard restriction enzyme digestion of aliquots using the restriction enzymes EcoRI plus NotI and AseI. All restriction enzyme digest fragments corresponded to the predicted sizes when analyzed on a 1% agarose gel.

[0041] Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4×10^6 cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% Hyclone Fetal Bovine Serum (FBS), 100U penicillin/100U streptomycin and glutamine (2mM). The following day each plate of cells was transfected with 6 μg of the pFlt1D2.Flk1D3.FcΔC1(a) plasmid DNA using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells, 12 ml/plate of Optimem supplemented with 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II supplemented with glutamine (2mM) and 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation, the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and purification of the expressed protein was performed as described of USSN 09/773,877.

Example 4: Construction pVEGFR1R2-FcΔC1(a) expression vector

[0042] The pVEGFR1R2.FcΔC1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of Fig. 24A-24C of USSN 09/773,877) between Flt1d2-Flk1d3-FcΔC1(a) amino acids 26 and 27 of Fig. 21A-21C of USSN 09/773,877 (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to

decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.FcΔC1(a) chimeric molecule is set forth in Fig. 24A-24C of USSN 09/773,877.

Example 5: Cell Culture Process Used to Produce Modified Flt1 Receptors

[0043] (a) Cell Culture Process Used to Produce Flt1D2.Flk1D3.FcΔC1(a). The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the expression plasmid pFlt1D2.Flk1D3.FcΔC1(a) described in Example 1 of USSN 09/773,877 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography. The process is provided in greater detail below.

[0044] Cell Expansion. Two confluent T-225 cm^2 flasks containing the Flt1D2.Flk1D3.FcΔC1(a) expressing cell line were expanded by passaging cells into eight T-225 cm^2 flasks in medium (GMEM + 10% serum, GIBCO) and incubated at 37°C and 5% CO_2. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm^2 roller bottles and incubated at 37°C and 5% CO_2 until confluent.

[0045] Suspension Culture in Bioreactors. Cells grown in roller bottles were trypsinized to detach them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5L of suspension culture. The suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25 μM methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37°C. When a density of 4 $x10^6$ cells/mL was reached the cells were transferred to a 40L bioreactor containing the same medium and

setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34°C to slow cell growth and increase the relative rate of protein expression.

[0046] (b) Cell Culture Process Used to Produce Flt1D2.VEGFR3D3.FcΔC1(a). The same methodologies as described *supra* for Flt1D2.Flk1D3.FcΔC1(a) were used to produce Flt1D2.VEGFR3D3.FcΔC1(a).

Example 6: Harvest and Purification of Modified Flt1 Receptors

[0047] (a) Harvest and Purification of Flt1D2.Flk1D3.FcΔC1(a). The product protein was aseptically harvested from the bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40L of harvest filtrate was then loaded onto a 400 mL column containing Protein A Sepharose resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. Flt1D2.Flk1D3.FcΔC1(a) protein was eluted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addition of Tris base and frozen at -20°C.

[0048] Several frozen lots of Flt1D2.Flk1D3.FcΔC1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30kD nominal molecular weight cutoff (NMWCO) tangential flow filtration membrane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL using a 30kD NMWCO membrane. The concentrated protein was loaded onto a size exclusion column packed with Superdex 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1D2.Flk1D3.FcΔC1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen.

[0049] (b) Harvest and Purification of Flt1D2.VEGFR3D3.FcΔC1(a). The same methodologies as described *supra* for Flt1D2.Flk1D3.FcΔC1(a) were used to harvest and purify Flt1D2.VEGFR3D3.FcΔC1(a).

Example 7: Phosphorylation Assay for Transiently Expressed VEGFR2

[0050] Primary human umbilical vein endothelial cells (HUVECs), passage 4-6, were starved for 2 hrs in serum-free DME high glucose media. Samples containing 40 ng/ml (1nM) human VEGF165, which is a ligand for the VEGF receptors Flt1, Flk1 and Flt4(VEGFR3) were prepared and were preincubated for 1 hr. at room temperature with varying amounts of the modified Flt1 receptors Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), Flt1D2Flk1D3.FcΔC1(a) and Flt1D2VEGFR3D3.FcΔC1(a) in serum-free DME-high glucose media containing 0.1% BSA. Cells were challenged for 5 minutes with the samples prepared above ± VEGF165, followed by whole cell lysis using complete lysis buffer. Cell lysates were immunoprecipitated with an antibody directed against the C-terminus of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto 4-12% SDS-PAGE Novex gel and then transferred to PVDF membrane using standard transfer methodologies. Detection of phosphorylated VEGFR2 was done by immunoblotting with the anti-phospho Tyrosine mAb called 4G10 (UBI) and developed using ECL-reagent (Amersham). The results of this experiment reveal that detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are phosphorylated to varying levels depending on which modified Flt1 receptor is used during the preincubations with VEGF. At a 1.5 molar excess of either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similarly, the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. Where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors. Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess or 3 and 4 fold molar excess of either transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or transient VEGFR1R2-FcΔC1(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to

control media challenge.

Example 8: Cell Proliferation Bioassay

[0051] The test cell population is MG87 cells that have been stably transfected with a expression plasmid that contains a DNA insert encoding the VEGFR2(Flk1) extracellular domain fused to the TrkB intracellular kinase domain, thus producing a chimeric molecule. The reason the TrkB intracellular kinase domain was used rather than the native VEGFR2(Flk1) intracellular kinase domain is that the intracellular kinase domain of VEGFR2(Flk1) does not cause a strong proliferative response when stimulated by VEGF165 in these cells. It is known that MG87 cells containing full length TrkB receptor give a robust proliferative response when stimulated with BDNF, so the TrkB intracellular kinase domain was engineered to replace the intracellular kinase domain of VEGFR2(Flk1) to take advantage of this proliferative response capability.

[0052] 5 x 10^3 cells/well were plated in a 96 well plate and allowed to settle for 2 hrs at 37°C. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40nM to 20pM and incubated on the cells for 1hr at 37°C. Human recombinant VEGF165 in defined media was then added to all the wells at a concentration of 1.56nM. The plates were incubated for 72 hrs at 37°C and then MTS (Owen's reagent, Promega) added and the plates were incubated for an additional for 4 hrs. Finally, the plates were read on a spectrophotometer at 450/570nm. The results show that control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) blocks 1.56nM VEGF165 with a half maximal dose of 0.8nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.FcΔC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~ 2nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

Example 9: Binding Stoichiometry of Modified Flt Receptors to VEGF165

[0053] (a) BIAcore Analysis. The stoichiometry of Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) surfaces or measuring

concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) to VEGF BIAcore chip surface.

[0054] Modified Flt receptors Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a), were captured with an anti-Fc specific antibody that was first immobilized on a Biacore chip (BIACORE) using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over the Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) surfaces at 10 μl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) molecule.

[0055] In solution, Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a concentration of 1nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured as a binding signal to an amine-coupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.FcΔC1(a) BIAcore binding signal to its molar concentration. The data showed that the addition of 1 nM VEGF165 into the Flt1D2Flk1D3.FcΔC1(a) solution completely blocked Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule. When the concentration of Flt1D2Flk1D3.FcΔC1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.FcΔC1(a) and -1,07 for VEGFR1R2-FcΔC1(a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a).

[0056] (b) Size Exclusion Chromatography. Flt1D2Flk1D3.FcΔC1(a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia Superose 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer

containing 6M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3.FcΔC1(a) was separated from VEGF165 using Superose 6 size exclusion chromatography column run in 6M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3.FcΔC1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under condition identical to one used in separating components of Flt1D2Flk1D3.FcΔC1(a)/VEGF complex. Quantification of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex composition was based on the calibration curves. The results of this experiment shows the ratio of VEGF165 to Flt1D2Flk1D3.FcΔC1(a) in a complex to be 1:1.

Example 10: Determination of the Binding Stoichiometry of Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography

[0057] Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex Preparation. VEGF165 (concentration = 3.61 mg/ml) was mixed with CHO cell transiently expressed Flt1D2.Flk1D3.FcΔC1(a) (concentration = 0.9 mg/ml) in molar ratio of 3:1 (VEGF165:Flt1D2.Flk1D3.FcΔC1(a)) and incubated overnight at 4°C.

[0058] (a) Size Exclusion Chromatography (SEC) under native conditions. To separate the complex from excess of unbound VEGF165, 50 µl of the complex was loaded on a Pharmacia Superose 12 PC 3.2/30 which was equilibrated in PBS buffer. The sample was eluted with the same buffer at flow rate 40µl/min. at room temperature. Peak #1 represents the complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl)was added to a final concentration 4.5M to dissociate the complex.

[0059] (b) Size Exclusion Chromatography (SEC) under dissociative conditions. To separate the components of the receptor-ligand complex and to determine their molar ratio, 50µl of dissociated complex as described *supra* was loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted with the same solution at a flow rate 40µl/min. at room temperature. The results are shown in Fig. 32 of USSN 09/773,877, with peak #1 Flt1D2Flk1D3.FcΔC1(a) and peak #2 VEGF165.

[0060] (c) Calculation of Flt1D2Flk1D3.FcΔC1(a):VEGF165 Complex Stoichiometry. The stoichiometry of the receptor-ligand complex was determined from the peak area or the peak height of the components. Concentrations of VEGF165 and Flt1D2Flk1D3.FcΔC1(a) corresponding to the peak height or peak area, respectively, were obtained from the standard curves for VEGF165 and Flt1D2Flk1D3.FcΔC1(a). To obtain a standard curve, four different concentrations (0.04 mg/ml - 0.3mg/ml) of either component were injected onto a Pharmacia Superose 12 PC 3.2/30 column equilibrated in 6M guanidinium chloride and eluted with the same solution at flow rate 40μl/min. at room temperature. The standard curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165:Flt1D2Flk1D3.FcΔC1(a) determined from the peak area of the components was 1.16. The molar ratio of VEGF165:Flt1D2Flk1D3.FcΔC1(a) determined from the peak height of the components was 1.10.

Example 11: Determination of the Stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography with On-Line Light Scattering

[0061] Complex preparation. VEGF165 was mixed with CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) protein in molar ratio of 3:1 (VEGF165:Flt1D2Flk1D3.FcΔC1(a)) and incubated overnight at 4°C.

[0062] (a) Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. Samples were injected onto a Superose 12 HR 10/30 column (Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. The elution profile showed two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300, the MW of VEGF165 at the peak position is 44 390 and the MW of R1R2 at the peak is 113 300.

[0063] These data indicated that the stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.FcΔC1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.FcΔC1(a).

Example 12: Peptide Mapping of Flt1D2.Flk1D3.FcΔC1(a)

[0064] The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify fragments with N-linked glycosylation sites. There were a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

[0065] There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation isobserved on Asn65 and Asn120.

Example 13: Pharmacokinetic Analysis of Modified Flt Receptors

[0066] (a) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a). Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-

3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8μg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the C_{max} was 18μg/ml and the C_{max} for the stable VEGFR1R2-FcΔC1(a) was 30μg/ml.

[0067] (b) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). The ELISA involves coating an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas , Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days or more.

Example 14: Evaluation of the Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit Tumor Growth *In Vivo*

[0068] To evaluate the ability of Flt1D2.Flk1D3.FcΔC1(a) to inhibit tumor growth in vivo a model in which tumor cell suspensions are implanted subcutaneously on the right flank of male severe combined immunodeficiency (SCID) mice was employed. Two cell lines, the human HT-1080 fibrosarcoma cell line (ATCC accession no. CCL-121) and the rat C6 glioma cell line (ATCC accession no. CCL-107), each of which exhibit distinctly different morphologies and growth

characteristics, were used in the assay. The first dose of Flt1D2.Flk1D3.FcΔC1(a) (at 25mg/Kg or as indicated in Figs. 1-2) was given on the day of tumor implantation. Animals subsequently received subcutaneous injections of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or vehicle either every other day (EOD) or two times per week (2X/wk) for a period of 2 weeks. After 2 weeks, animals were perfused with fixative, tumors were removed and samples were blinded. Tumor volume was determined by measuring the length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc (A40) and Flt1D2.Flk1D3.FcΔC1(a) significantly reduced the growth of tumors formed by HT-1080 and C6 cells.

Example 15: The Effect of VEGF165 and Modified Flt Receptors in Female Reproductive System

[0069] The stereotypic pattern of vascular remodeling which occur in the uterus and ovary over the course of the reproductive cycle has been well characterized, making these tissues particularly well suited to the study of mechanisms which regulate angiogenesis, vascular remodeling and vascular regression. Indeed, *in situ* hybridization studies in the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in mature rodents, as well as humans and non-human primates. As cyclic angiogenesis and vascular remodeling are prominent features of the normal ovary and uterus, it is not surprising that abnormal blood vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these organs. Furthermore, these pathogenic vascular abnormalities are thought to be caused or perpetuated by the dysregulated expression of one or more angiogenic or anti-angiogenic factors, most prominently VEGF.

[0070] Abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial carcinoma, and in each case VEGF is over expressed in the affected tissue. Overexpression of VEGF is also thought to play a pathogenic role in the establishment of systemic vascular hyperpermeability in ovarian hyperstimulation syndrome. In addition, VEGF has been implicated as the permeability factor responsible for the production of ascites associated with ovarian carcinoma and other tumors. Agents which effectively neutralize the biological actions of VEGF can reasonably be anticipated to be of therapeutic benefit in the above and related conditions.

[0071] Angiogenesis and vascular remodeling are also hallmarks of blastocyst implantation and placental development. VEGF is strongly expressed both in the maternal decidua and in embryonic trophoblasts, where it is thought to first stimulate expansion and hyperpermeability of the uterine vasculature during the peri-implantation period and subsequently mediate formation of both the maternal and embryonic components of the placental vasculature. VEGF is also required for luteal angiogenesis and associated progesterone secretion necessary to prepare the uterus for implantation. Thus, agents which inhibit the biological actions of VEGF may prove to be useful as contraceptive agents (by preventing implantation), or as an abortifacients in the early stages of gestation. The latter application might find particular use as a non-surgical intervention for the termination of ectopic pregnancies.

[0072] While the expression of VEGF receptors is largely confined to the vascular endothelium in normal reproductive tissues, Flt1 is also expressed by trophoblasts in the placenta in both humans and animals where it has been proposed to play a role in trophoblast invasion. Interestingly, both Flt1 and KDR (Flk1) are expressed by choriocarcinoma cell line BeWo, and VEGF has been shown to promote DNA synthesis and tyrosine phosphorylation of MAP kinase in these cells. Furthermore, primary and metastatic ovarian carcinomas not only to express high levels of VEGF, but - in addition to the vascular endothelium - the tumor cells themselves express KDR and/ or Flt1. These findings suggest that VEGF may not only be critically involved in the generation and maintenance of tumor vasculature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly supporting the survival and proliferation of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of reproductive origin.

[0073] Assessment of VEGF-Induced Uterine Hyperpermeability. Pregnant mare's serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction results in hyperpermeability of the uterus and an increase in uterine wet weight 6 hrs. later and, therefore, could potentially be blocked by the modified Flt receptors Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). In this in vivo model, the normal weight of the rat uterus is about 50

mg and this can be induced to 300-350 mg by PMSG. Desiccation of the tissue reveals that this is all water weight. Subcutaneous injection of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at 1hr. after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight. Increasing the dose of modified Flt receptor does not further reduce the increase in wet weight suggesting that there is a VEGF-independent component to this model. The results of this experiment are shown in Fig. 3.

[0074] Assessment of corpus luteum angiogenesis using progesterone as a readout. Pregnant mare's serum gonadotrophin (PMSG) is injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a fully functioning corpus luteum containing a dense network of blood vessels after 4 days that allows for the secretion of progesterone into the blood stream in order to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF; therefore, blocking VEGF would result in a lack of new blood vessels and thus a lack of progesterone secreted into the blood stream. In this in vivo model, resting levels of progesterone are about 5ng/ml and this can be induced to a level of 25-40ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at 1hr. after PMSG injection results in a complete inhibition of the progesterone induction on day 4. The results of this experiment are shown in Figs. 4A-B.

Example 16: Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc

[0075] Flt1(1-3)-Fc was PEGylated with either 10kD PEG or 20kD PEG and tested in balb/c mice for their pharmacokinetic profile. Both PEGylated forms of Flt1(1-3)-Fc were found to have much better PK profiles than Flt1(1-3)-Fc (A40), with the Tmax occurring at 24 hrs. for the PEGylated molecules as opposed to 6 hrs. for Flt1(1-3)-Fc (A40).

Example 17: VEGF165 ELISA to Test Affinity of Modified Flt1 Receptor Variants

[0076] 10pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160pM to 0.1pM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-FcΔC1(a), Flt1-(1-3_{NAS})-Fc, Flt1(1-$3_{R->C}$)-Fc and Tie2-

Fc. Flt1(1-3 $_{NAS}$)-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR is replaced by NASVNGSR, resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-3 $_{R->C}$)-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) KNKCASVRRR (SEQ ID NO:18) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1-$3_{R->C}$)-Fc, Flt1(1-3_{NAS})-Fc and Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

Example 18: The effects of VEGFR1R2-FcDC1(a) in a novel animal model of psoriasis.

[0077] K14VEGF Transgenic mice. A Keratin-14 (K14)-based expression vector and a mouse cDNA encoding VEGF164 was used to generate K14VEGF transgenic mice by an approach identical to that used for generating K14-Angl mice (Suri et al. (1998) Science 16;282(5388):468-71). The K14 promoter directs expression of VEGF to the basal layer of the epidermis, including cells lining the hair. The K14VEGF transgenic homozygous mice were used throughout the studies described herein.

[0078] Tissue processing and immunostaining. For immunohistochemistry, 10μm cryo-sections of ear skin obtained from both wild-type and K14VEGF transgenic mice were stained with anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31, PharMingen, San Diego, CA), anti-mouse CD4 (BD PharMingen, San Diego, CA), CD8 (BD PharMingen, San Diego, CA), anti-mouse F4/80 (Serotec, Oxford, England), or anti-mouse VEGF (R&D systems, Minneapolis, MN). For immunostaining with PECAM-1 and VEGF, tissue sections were pre-fixed in 4% paraformaldehyde before staining according to standard procedure known in the art. For

immunostaining with CD4, CD8, or F4/80 antibody, acetone-fixed tissue sections were used that were prepared by standard techniques familiar in the art.

[0079] Histology. Hematoxolin and eosin (H&E) staining were performed according to standard protocols familiar to the skilled artisan.

[0080] VEGFR1R2-FcDC1(a) injection. The K14VEGF homozygous transgenic mice were treated with VEGFR1R2-FcDC1(a) by subcutaneous injection into the neck skin. The mice were treated with either 25 mg/kg VEGFR1R2-FcDC1(a) or 12.5 mg/kg human Fc as a control, using an injection schedule of every three days for 10 days resulting in a total of four injections per animal. Photographs of the mice were taken immediately before each injection. Mouse ear tissue was harvested on day 12 for subsequent histological analyses.

[0081] Phenotype of K14VEGF transgenic mice. As previously reported, the K14VEGF transgenic mice are fertile and overtly healthy (Suri et al. (1998) *supra*; Detmar et al. (1998) *supra*). However, the ear skin of the K14VEGF transgenic mice is visibly redder than that of their wild-type FVB littermates. Focal lesions which appeared similar to psoriatic lesions started to develop on the ear skin and, to a lesser extent, on the dorsal and lateral skin of young K14VEGF transgenic mice. The condition worsened with age. Massive skin lesions were observed on the ears of these transgenic mice by age 5 months or older. Lesions were accompanied by bloody, flaky skin, and hair loss. Fig. 5 is a photograph of the mice exhibiting such massive lesions at about 6 months of age.

[0082] Expression of VEGF in the skin of K14VEGF transgenic mice. VEGF transgene expression was detected by immunostaining with an antibody specific to mouse VEGF. Strong protein expression was observed in basal keratinocytes and in microvessels in the papillary dermis.

[0083] Histological analyses of psoriatic lesions in K14VEGF transgenic mice. Histological analyses of K14VEGF transgenic mouse ears exhibiting the psoriatic lesions revealed a characteristic psoriatic skin phenotype. Standard hematoxolin and eosin (H&E) staining revealed that the epidermis of young K14VEGF transgenic mice exhibited moderate acanthosis (i.e. epidermal hyperplasia) and focal parakeratosis (i.e. keratinocytes in the stratum corneum retain nuclei) compared to control mouse (see Figs. 6A and B). In the dermal compartment, edema coupled with an approximately 5-fold increase in tissue thickness was observed, as was inflammatory cell infiltration. The condition progressed with age. K14VEGF transgenics over 6 months of age

developed obvious rete ridges that are typical for psoriasiform hyperplasia and the skin became more thickened (see Fig. 7). More confluent hyperkeratosis with excessive deposition of keratin, and parakeratosis with neutrophil-laden pustules were present in the stratum corneum (Fig. 6C). Munro microabscesses (Fig. 8A) localized within parakeratotic areas of the cornified layer (Altman et al. (1999) *supra*) and Kogoj microabscesses (Fig. 8B) that localized immediately beneath the parakeratotic cornified layer were identified in the lesions of older K14VEGF transgenics. The presence of microabscesses are key features in clinical psoriasis diagnosis.

[0084] K14VEGF transgenic mice are characterized by visible skin redness and vascularization. Immunohistological staining for PECAM-1, an integral membrane protein located on endothelial cells (DeLisser et al. (1994) Immunol. Today Oct;15(10):490-5) revealed an increased number of dermal microvessels within K14VEGF transgenic skin. Dilated and tortuous capillaries in the papillary dermis, that spiral to near the undersurface of the epidermis, were also observed.

[0085] The pathological basis for psoriasis is not known. One issue is whether the disorder reflects an abnormality in the epidermal keratinocyte or bone-marrow-derived immunocytes. Recent studies using severe combined immunodeficient (SCID) mice engrafted with symptomless skin from a psoriasis patient provided direct in vivo evidence that activated CD4+, but not CD8+ T-lymphocytes, can trigger the formation of a psoriatic phenotype (Wrone-Smith et al. (1996) J. Clin. Invest. Oct 15;98(8):1878-87; Nickoloff et al. (1999) Am. J. Pathol. Jul;155(1):145-58). To analyze the immunologic basis that mediates the inflammatory response in the K14VEGF transgenic mouse psoriasis model described herein, immunostaining was performed for CD4+ and CD8+ immunocytes. The results revealed massive infiltration of CD4+ T-lymphocytes that are localized primarily in the dermis of both early psoriatic lesions and in more mature psoriatic lesions isolated from older K14VEGF transgenic mice. The overall level of CD8+ T-lymphocytes that infiltrated into the lesional skin was significantly less than that of the CD4+ T-lymphocytes. In young K14VEGF transgenic lesions, CD8+ T-lymphocytes were detected in both the dermis and the epidermis. Interestingly, CD8+ lymphocytes become primarily localized in the epidermis with maturation of psoriatic lesion. When cryosections of skin from K14VEGF transgenic mice were stained with an antibody recognizing the murine macrophage marker F4/80 antigen, a significant increase in the number of macrophages was observed as compared to control. This increased macrophage

infiltration became even more dramatic with the development of psoriatic lesions in older transgenic mice, which suggests that the cytokines or growth factors secreted by activated CD8+ lymphocytes further stimulate macrophage proliferation leading to exacerbation of psoriatic phenotype.

[0086] The effects of VEGFR1R2-FcDC1(a) in an animal model of psoriasis. The novel animal model of psoriasis described herein demonstrates that a psoriatic phenotype can be induced primarily by over expression of VEGF in the mouse epidermis. To confirm the causative role of VEGF in the formation of a psoriatic lesion, VEGFR1R2-FcDC1(a) was injected subcutaneously into mouse neck skin. VEGFR1R2-FcDC1(a) competes with endogenous mouse VEGF receptor for binding of VEGF by forming a complex with the VEGF, thus preventing it from binding to its receptor and transducing a signal. Five K14VEGF transgenic mice with obvious psoriatic lesions were treated on days 0 (Figs. 9A-D), day 3 (Figs. 9E-H), day 7 (Figs. 9I-L), and day 10 (Figs. 9M-P), with VEGFR1R2-FcDC1(a) at a dose of 25 mg/kg. Three of the treated mice showed significant improvement of the skin lesions by day 3, following the first injection of VEGFR1R2-FcDC1(a). The remaining two of the animals showed mild improvement in their lesions by day 3. Subsequent injections of VEGFR1R2-FcDC1(a) demonstrated further improvements in the skin lesions in all the mice up to day 7. However, by day 10, two of the mice started to develop small focal lesions, presumably due to the formation of VEGFR1R2-FcDC1(a) neutralizing antibodies (Figs. 9A-P).

Example 19: The effects of VEGFR1R2-FcDC1(a) in a novel animal model of wound healing

[0087] During the early phase of wound healing, new granulation tissue begins to form approximately 4 days after the injury. Numerous new capillaries along with fibroblasts and extracellular matrix proteins move into the wound space (Hunt (1980) World J. Surg. 4(3):271-7). Neo-vascularization provides oxygen and nutrients necessary to sustain cell metabolism. In fact, adequate new blood vessel formation seems to be crucial to the normal process of wound healing. However, the growth factor(s) that stimulate the angiogenesis associated with wound healing as well as the underlying molecular mechanisms at play remain elusive.

[0088] VEGF, a potent angiogenesis factor, has strong vasopermeability activity (Dvorak et. al. (1995) Am. J. Pathol. 146(5):1029-39) and is produced in large quantities by the epidermis during

wound healing (Brown et al. (1992) J. Exp. Med.1;176(5):1375-9). Therefore, the role of VEGF in wound healing using a novel murine excisional wound healing model was studied.

[0089] Murine excisional wound healing model. A novel wound healing model was created by introducing an excisional wound on the dorsal skin of a mouse ear. Female FVB mice (Taconic, NY) weighing approximately 25 to 30g were used in this experiment. Animals were housed under standard conditions, and provided food and water ad libitum. Post-operatively, animals were housed in individual cages under standard conditions, and checked daily for signs of healing. Mice were anesthetized using ketamine (200 mg/kg) and xylazine (10 mg/kg) through intraperitoneal injection. Using electric clippers, the hair on the ear skin of the mice was gently shaved. A standard depilating agent was applied to remove the remaining hair, and PBS and betadine was used to clean the exposed skin. One full-thickness, circular wound was created on each ear. Excision was made by 4 mm biopsy punch (Clark, NY) extending down to bare cartilage, followed by dissection with a microknife (Roboz, MD). Nicks were made on ear cartilage to mark the origin of wound. All the wounds were covered with an occlusive polyurethane dressing (Tegaderm, 3M, Minneapolis, MN until harvest. Upon harvest, animals were euthanized by lethal intraperitoneal injection of ketamine and xylazine. The wounds were bisected and analyzed histologically. These wounds, splinted by underlying cartilage, were minimally Re-epithelialization rate, percentage of full re-epithelialization, and new granulation tissue formation in all age and sex matched wounds were measured by computer assisted image analysis program Osteomeasure (Osteometrics, Inc. Atlanta, GA). Tissue preparation, Histology, and VEGFR1R2-FcDC1(a) injections were done as described *supra* in Example 6.

[0090] Wound healing in K14VEGF transgenic mice. One wound was introduced onto each ear of homozygous K14VEGF transgenic mice. Wounds were harvested on days 3, 7 and 10 after surgery. Cryosections were stained with H&E for morphological analysis. Wound tissue was quantified for granulation tissue formation and neo-epithelialization using a computer-assisted imaging program Osteomeasure (Osteometrics, Inc. Atlanta, GA). In 3 month old K14VEGF transgenic mice, granulation tissue formation was impaired by 37.8% ($p<0.05$) on POD3 compared to age-matched wild type littermate control mice.

[0091] The effects of VEGFR1R2-FcDC1(a) on wound healing in normal mice. As stated *supra*, overexpression of VEGF in mouse skin retards wound healing, presumably due to an

excessive inflammatory response and edema. However, in this experiment, blocking endogenous VEGF in normal FVB mouse wounds by administering VEGFR1R2-FcDC1(a) at 25 mg/kg does not affect wound healing in a significant way. This may be due to the relatively lower levels of VEGF in a "normal" wound as compared to the high levels of expression in chronic wounds which tend not to heal efficiently. These observations support the use of VEGFR1R2-FcDC1(a) in improving and enhancing wound healing in clinical settings in which VEGF is overexpressed by down regulating inflammation and edema.

[0092] The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof.